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TITLE: Atypical Opioid Mechanisms of Control of Injury-Induced Cutaneous Pain by Delta Receptors

PRINCIPAL INVESTIGATOR: Dr. Gregory Scherrer

CONTRACTING ORGANIZATION: Stanford University, Palo Alto, CA 94304

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Board of Trustees of the Leland
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Alto, CA 94304-5589

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Severe pain due to war-related injuries is difficult to treat, and current opioids (i.e. mu opioid receptor agonists such as morphine) cause unacceptable side effects including addiction. Injuries suffered most frequently by active military personnel include traumatic brain injury, nerve trauma, skin incision, and burn injury, and all these injuries are associated with acute cutaneous pain and/or mechanical allodynia/hypersensitivity. The goals of our research are to evaluate analgesics acting on delta opioid receptors (DORs) in animal models relevant to today's battlefield experience (Specific Aim 2), and elucidate the mechanisms by which DOR agonists, administered in skin and acting on mechanosensory dorsal root ganglia neurons, relieve pain (Specific Aim 1). We have determined the analgesic effect of two DOR agonists, deltorphin II and SNC80. We show that these compounds significantly elevate mechanical pain threshold, indicating their acute antinociceptive action. Furthermore, we found that in two models of injuries, namely skin incision and nerve trauma, a single injection of deltorphin II eliminates the mechanical hyper sensitivity caused by injury. We have also initiated studies aiming at identifying the peripheral sensory neurons that express DOR, a first step towards understanding the analgesic mechanism of action of DOR agonists. We are currently extending these findings by performing the other experiments described in our original proposal, without significant change in our plans and strategy. Importantly, our promising results support our hypothesis that DOR agonists, acting in the skin, represents an effective therapeutic strategy for blocking severe pain associated with injuries that can be suffered on the battlefield.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	18	

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Intense pain resulting from injuries suffered on the battlefield is difficult to treat, and current opioids that act on mu opioid receptors such as morphine generate significant side effects including addiction. War-related injuries suffered most frequently by military personnel include traumatic brain injury, nerve trauma, skin incision, and burn injury, and all these injuries are associated with acute cutaneous pain and/or mechanical allodynia. In this research we evaluate analgesics acting on delta opioid receptors (DORs) in animal models relevant to today's battlefield experience, and elucidate the mechanisms by which DOR agonists, administered in skin and acting on mechanosensory dorsal root ganglia neurons, relieve pain.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Delta opioid receptor (DOR) agonists, cutaneous pain, acute pain, injuries suffered on the battlefield, injury-induced chronic pain, burn injury, incision injury, nerve injury, analgesia, mouse, intraplantar injections, pain behavior, electrophysiology, dorsal root ganglion neurons, histology, mechanism of action

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 2 –To test the hypothesis that peripheral administration of DOR agonists can reduce acute cutaneous pain and chronic mechanical allodynia in rodent models of injuries that soldiers can suffer on the battlefield.

Major Task 3: Acute pain
Subtask 1: To evaluate the utility of DOR agonists for the treatment of acute cutaneous pain. Months 6-24
Estimated percentage of completion: 50%
Estimated time for completing Milestone #4 Demonstration of the efficacy of DOR agonists to reduce acute pain: 24 months (no change)

Major Task 4: Injury-induced mechanical allodynia
Subtask 1: To evaluate the utility of DOR agonists for the treatment of nerve trauma-induced mechanical allodynia.
Months 6-30
Estimated percentage of completion: 50%

Subtask 2: To evaluate the utility of DOR agonists for the treatment of mechanical allodynia induced by incision injury.
Months 1-24
Estimated percentage of completion: 50%

Subtask 3: To evaluate the utility of DOR agonists for the treatment of burn injury-induced mechanical allodynia.
Months 12-36
Percentage of completion: 0%

Estimated time for completing Milestone #5 Demonstration of the efficacy of DOR agonists to reduce injury-induced mechanical allodynia: 30 months (6 months earlier than originally the 36 months originally scheduled, as we put more effort towards Major Task 4 because we could not begin experiments for Major Tasks 1 & 2)

Specific Aim 1 – To resolve the mechanism of action by which peripherally administered DOR agonists inhibit neuronal activity.

Major Task 1: Electrophysiological analysis
Subtask 1: To establish the effect of DOR agonists on the activity of mechanosensitive DRG neurons using a largely intact ex vivo somatosensory system preparation
Months 1-30
Percentage of completion: 0%
Estimated time for completing Milestone #1 Demonstration that DOR agonists inhibit action potential firing in cutaneous mechanosensitive DRG neurons: 36 months (6 months later than the 30 months originally scheduled, due to delays in obtaining approval for our animal protocol and starting these experiments)

Subtask 2: To resolve the molecular mechanisms by which DOR agonists inhibit DRG neurons in primary culture.
Months 6-30
Percentage of completion: 0%
Estimated time for completing Milestone #2 Resolution of the mechanism of action of DOR agonists: 36 months (6 months later than the 30 months originally scheduled, due to delays in obtaining the electrophysiological equipment for performing these experiments)

Major Task 2: Characterization of DOR-expressing neurons in human DRG
Subtask 1: To characterize DOR-expressing neurons in human DRG by in situ hybridization
Months 12-24
Percentage of completion: 10%
Estimated time for completing Milestone #3 Demonstration that DOR expression in human DRG is similar to that observed in mouse: 24 months (no change)

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1. *Major Task 1: Electrophysiological analysis*
   Subtask 1: To establish the effect of DOR agonists on the activity of mechanosensitive DRG neurons using a largely intact ex vivo somatosensory system preparation.
   Subtask 2: To resolve the molecular mechanisms by which DOR agonists inhibit DRG neurons in primary culture.

   We are breeding DORGFP mice to perform these experiments

2. *Major Task 2: Characterization of DOR-expressing neurons in human DRG*
   Subtask 1: To characterize DOR-expressing neurons in human DRG by in situ hybridization

   Tissues used: human dorsal root ganglia (DRG) [National Disease Research Interchange (NDRI)]

   We have started these experiments. We cryoprotected DRG received from NDRI in sucrose 30%, embedded DRG in O.C.T., and froze DRG. We next sectioned DRG at 20 microns with a cryostat and applied cryosections on slides. Slides were then processed for fluorescent in situ hybridization with RNAscope technology (ACD Biosystems) to detect Oprd1 mRNA, as described previously for mouse DRG (Bardoni et al., Neuron, 2014). A number of controls were used including positive controls (Oprd1 probe on mouse tissue as done in Bardoni et al., Neuron, 2014) and negative controls (no probe).

   Controls indicated that the technology and reagents work as expected and allow the specific detection of Oprd1 mRNA in tissues. However, the controls also indicated that a high level of endogenous fluorescence was present in human DRG sensory neurons (Figure 1). This high level of endogenous fluorescence prevents the visualization of fluorescently labelled Oprd1 mRNA. To circumvent this problem, we are currently developing alternate in situ hybridization protocols. We are using two alternate approaches: 1. A non-fluorescent in situ hybridization protocol, and 2. Protocols for eliminating the endogenous fluorescence before processing the DRG sections for fluorescent in situ hybridization. Specifically we are evaluating the utility of TrueBlack™ (Biotum), a new reagent for quenching lipofuscin autofluorescence in tissue sections for immunofluorescence staining.

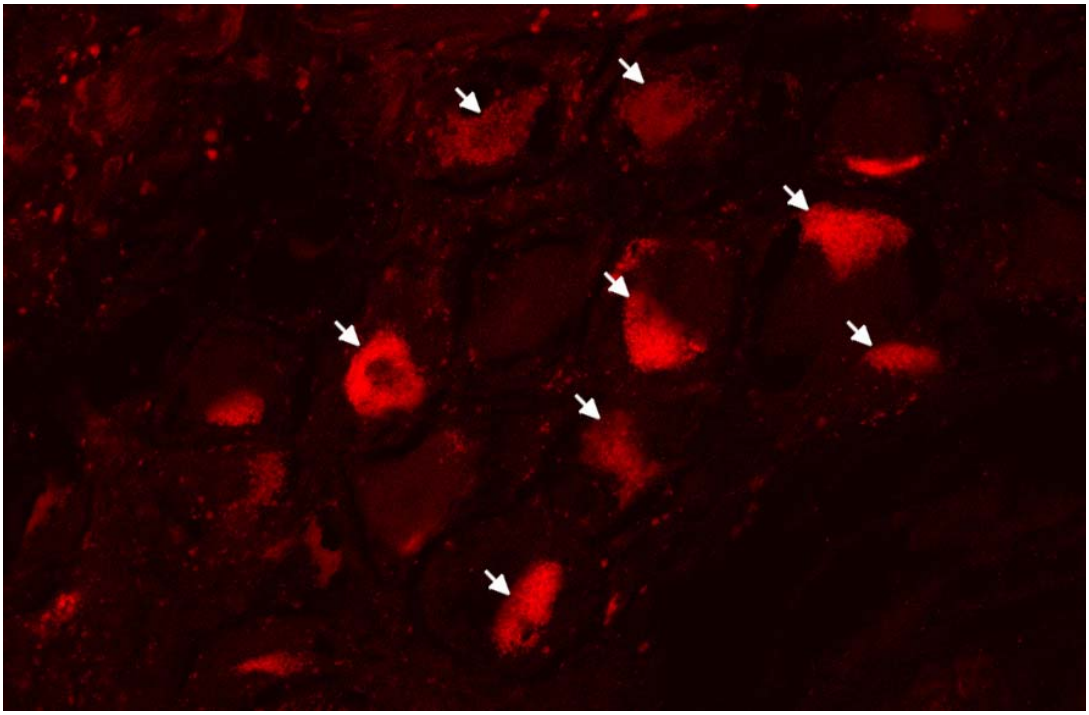

Figure 1. Expression of DOR in sensory neurons in human DRG (Major Task 2, Subtask 1). Image of a section of human DRG processed for fluorescent in situ hybridization to detect Oprd1 mRNA. The high level of endogenous fluorescence prevents the visualization of fluorescently labelled Oprd1 mRNA. Arrows indicate DRG neuron somata with endogenous fluorescence.

3. *Major Task 3: Acute pain*
   Subtask 1: To evaluate the utility of DOR agonists for the treatment of acute cutaneous pain.

   We have started behavioral experiments in mice to evaluate the utility of DOR agonists for the treatment of acute cutaneous pain. As proposed in our application, we tested the effect of the high affinity DOR agonists deltorphin II and SNC80 on mechanical sensitivity, using the von Frey test. The experiments were performed as described previously (Scherrer et al., PNAS, 2010; Scherrer et al., Cell, 2009). Briefly, mice were placed in plastic chambers on a wire mesh grid 1 hour before testing for habituation. Mice were then injected in the plantar surface with 10 microliters of either a control vehicle solution or deltorphin II (10 micrograms) or SNC80 (25 micrograms), and stimulated with von Frey filaments (North Coast Medical Inc., Morgan Hill, CA, USA) according to the up-down method (Chaplan et al., J Neurosci Methods, 1994), starting with 0.1 g and ending with 2.0 g filament as cutoff value. As shown in Figure 2, our preliminary experiments suggest that the intraplantar administration of deltorphin II or SNC80 increase the mechanical threshold and thus diminishes mechanical sensitivity, as hypothesized. We are presently continuing these experiments, using additional doses of agonists, and testing the specificity of these effects in DOR knockout mice.

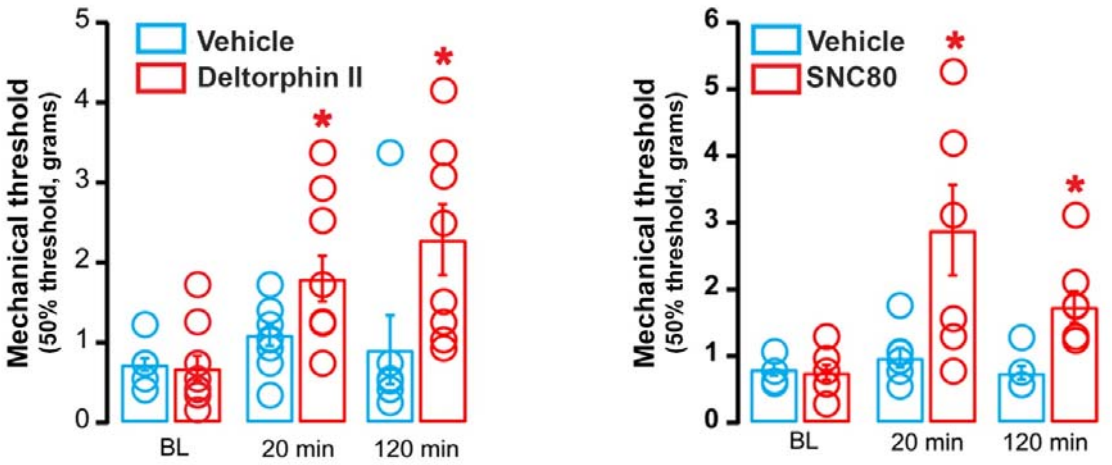

*Figure 2. Effect of the DOR agonists deltorphin II and SNC80 on mechanical sensitivity (Major Task 3, Subtask 1). Intraplantar injection of deltorphin II (10 micrograms) or SNC80 (25 micrograms) increased mechanical threshold measured with von Frey filaments (50% threshold: threshold at which a response is observed 50% of the time following stimulation, as in the up-down method described by Chaplan et al., J Neurosci Methods, 1994) 20 and 120 minutes after administration, compared to baseline (BL). These promising results suggest that DOR activation in the periphery can reduce mechanical pain, as hypothesized. Data are represented as mean + SEM (error bars). Values obtained for each mouse tested are indicated by the empty circles. Statistical analysis used a Repeated Measures ANOVA and Bonferroni posthoc test. * indicates $p<0.05$.*

Major Task 4: Injury-induced mechanical allodynia
Subtask 1 To evaluate the utility of DOR agonists for the treatment of nerve trauma-induced mechanical allodynia.
Subtask 2: To evaluate the utility of DOR agonists for the treatment of mechanical allodynia induced by incision injury.

We have initiated behavioral studies to determine whether intraplantar injection of deltorphin II can reduce the mechanical hypersensitivity resulting from injuries, including skin incision, nerve trauma, or burn. For the hindpaw incision model of incisional injury, mice were anesthetized using isoflurane (2% in O2, 2 L/min) delivered through a nose cone. After sterile preparation with betadine, a 5-mm longitudinal incision was made with a number 11 scalpel blade on the plantar surface of the right hindpaw. The incision is sufficiently deep to divide deep tissue including the plantaris muscle longitudinally. After controlling any bleeding, a single 6-0 nylon suture was placed through the midpoint of the wound and antibiotic ointment was applied. Figure 3 shows that this procedure caused cutaneous mechanical hypersensitivity for several days, as measured with the von Frey test. Remarkably, a single intraplantar injection of deltorphin II was sufficient to reverse this hypersensitivity. We are presently completing these studies, using additional doses and injury models, as proposed in our application. Our preliminary results indicate that intraplantar deltorphin II also relieves pain resulting from nerve trauma, while studies with the burn injury model will be debuted soon. Collectively, these very encouraging data support our claim that activation of peripheral DORs is an efficient therapeutic strategy for reducing cutaneous mechanical pain that results from injuries that can be suffered on the battlefield.

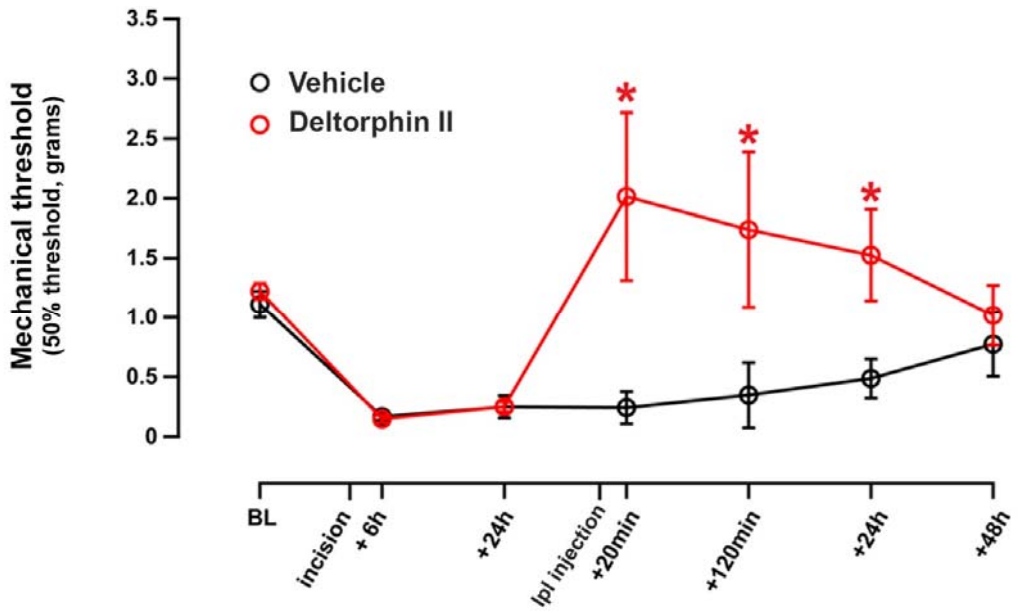

*Figure 3. Effect of the DOR agonists deltorphin II on mechanical hypersensitivity induced by incision injury (Major Task 4, Subtask 2). Incision at the level of the hindpaw reduced mechanical pain threshold (i.e. indicating cutaneous pain) compared to baseline (BL), as soon as 6 hours after injury, and for several days. A single intraplantar injection of deltorphin II (10 micrograms) 24 hours after injury increased mechanical threshold and essentially eliminated cutaneous hypersensitivity. These results suggest that DOR activation in the periphery efficiently limits hypersensitivity resulting from incision. Data are represented as mean + SEM (error bars). Statistical analysis used a Repeated Measures ANOVA and Bonferroni posthoc test. * indicates $p<0.05$.*

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report. The project was not intended to provide training and professional development opportunities.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

We are still collecting data to confirm our initial findings and have not yet debuted to disseminate knowledge acquired from this study.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

During the next reporting period, we will pursue Specific Aim 2 behavioral studies (Major Tasks 3 and 4), using additional doses and ligands (SNC80) and models of injury (burn injury), as well as develop Specific Aim 1 histological studies aiming at identifying DOR-expressing sensory neurons (Major Task 2), as described in the original SOW. We will also initiate electrophysiological studies (Specific Aim 1, Major Task 1) to resolve the analgesic mechanism of action of DOR agonists, again following the original SOW.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

At this stage, our initial promising results have to be confirmed and solidified with the additional experiments and approaches, as described in our proposal, before evaluating the impact of the project on its principal discipline.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Similarly, it is too early to evaluate the impact of the project on other disciplines.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report. It is too early to evaluate the impact of the project on other disciplines.

the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report. It is too early to evaluate the impact of the project on society.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We do not have any changes to Report.

We do have one problem to Report.

1. Need to purchase Piezo actuator and controller for performing experiments of Major Task 1, Subtask 2: Electrophysiological analysis to resolve the molecular mechanisms by which DOR agonists inhibit DRG neurons in primary culture. However the items were originally listed as supplies on the itemized budget. The items are in fact pieces of equipment, by Stanford policy. Awaiting approval from Sandra Rosario, Grant Specialist, before we move forward with the order.

Actual or anticipated problems or delays and actions or plans to resolve the
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We do not anticipate significant problems.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

We are still collecting data to confirm our initial findings and have not yet submitted any article for publication.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

We are still collecting data to confirm our initial findings and have not yet published any book, monograph, dissertation, abstract, or the like published as or in a separate publication

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	***Dr. Gregory Scherrer***
Project Role:	***PI***
Researcher Identifier (e.g. ORCID ID):	*gscherrer (eRA Commons)*
Nearest person month worked:	*4.8*
Contribution to Project:	Dr. Scherrer has overall responsibility for the proposed research. Specifically, he designs the proposed experiments, and analyze data and interpret the results.
Funding Support:	*Department of Defense; Anesthesia Department*
Name:	***Amaury Francois***
Project Role:	***Post-Doctoral fellow***
Researcher Identifier (e.g. ORCID ID):	*francois.amaury (eRA Commons)*
Nearest person month worked:	*9*
Contribution to Project:	Dr. François performs, analyzes, and interprets in situ hybridization studies (Specific Aim 1, subaim 1.b.), and behavioral experiments (Specific Aim 2), and electrophysiological experiments in DRG primary culture (Specific Aim 1, subaim 1.b.), Dr. François also presents the results to the research group.
Funding Support:	*Department of Defense; Start-up funds from Dr. Scherrer*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: None

9. APPENDICES: None